RESEARCH PAPER

Adolescent sleep timing and dietary patterns in relation to DNA methylation of core circadian genes: a pilot study of Mexican youth

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ABSTRACT

Mistimed sleep/wake and eating patterns put shift workers at increased risk for cardiometabolic disease, and epigenetic modification of circadian genes has been proposed as a mechanism. Although not as extreme as shift workers, adolescents often have delayed sleep timing and irregular eating patterns. The aim was to assess whether sleep midpoints – median of bed and wake time – and dietary patterns in adolescents were associated with DNA methylation of circadian genes. The study population included 142 Mexican youth (average age of 14.0 (SD = 2.0) years, 49% male). Average sleep midpoint over weekdays was estimated with actigraphy. Diet was assessed with a semi-quantitative food frequency questionnaire, and three dietary patterns were derived from principal component analysis, a *Plant-based & lean proteins* pattern, a *Meat & starchy* pattern, and an *Eggs, milk & refined grain* pattern. DNA methylation was quantified in blood leukocytes with the Infinium MethylationEPIC BeadChip, and data from 548 CpG sites within 12 circadian genes were examined. Linear regression analyses, adjusted for sex, age, and % monocytes, showed that later sleep timing was associated with higher DNA methylation of several circadian genes, notably with *RORB*, *PER1*, *CRY2*, and *NR1D1*. Each of the dietary patterns examined was also related to circadian gene DNA methylation, but the *Eggs, milk & refined grain* pattern ('breakfast' pattern) had the clearest evidence of relationships with circadian genes, with inverse associations (lower DNA methylation) across all 12 genes. Findings suggest that timing-related sleep and eating behaviours among adolescents could result in epigenetic modification of clock genes.

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Introduction

Circadian disruption refers to a misalignment in timing of daily activities, including eating and sleeping, with the internal circadian rhythm. Shift workers display the most extreme example of circadian disruption, but there are a range of circadian disruptions present in non-shift work populations. Adolescents are one population who may be particularly susceptible to circadian disruption, since there are multiple factors that delay bedtimes (e.g., social media and screen use [1]) and increase variability in sleep timing [2] (e.g., sleeping in late on weekends, napping during the day). Eating patterns may also contribute to misalignment of daily activities among

adolescents, as the pubertal transition often coincides with changes in eating behaviours, particularly with alterations in timing (e.g., late-night eating [3]) and stability [4,5] (e.g., irregular snacking rather than regular meals).

Research among shift workers consistently shows that sleeping and eating at non-optimal times is associated with cardiometabolic disease risk [6,7]. Moreover, mounting evidence suggests that circadian disruption observed within non-shift work populations, including adolescents, may also increase risk for cardiometabolic disease. For example, sleep timing (a typical proxy for circadian disruption), independent of sleep duration, was associated with higher metabolic syndrome risk among girls in a cohort of adolescents from the US [8]. In addition,

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a study of adolescents from Morelos, Mexico showed that both later (after 11 PM) and earlier (before 9 PM) bedtimes, as compared to those between 9 and 10 PM, were associated with higher incidence of elevated blood pressure over a 14-month period [9].

One proposed mechanism to explain a link between disrupted circadian rhythms and poor cardiometabolic health is through altered expression of circadian genes. Circadian, or clock genes, are responsible for maintaining 24-h rhythmicity of sleep and wake processes, including the diurnal rhythms of metabolism [10]. One pathway by which gene expression can be altered by environmental cues such as sleep and diet is through the epigenetic mechanism of DNA methylation [11,12], i.e., the addition of a methyl group to the 5'-carbon of cytosine in a Cytosine-phosphate-Guanine (CpG) dinucleotide. Recent evidence shows that shift workers in comparison to those on day shifts have differential DNA methylation patterns of certain circadian genes [13,14], which in turn is likely related to differences in gene expression and function [15]. Nonetheless, the degree to which circadian disruption could alter DNA methylation of circadian genes within adolescent populations remains unknown. Adolescence represents an important developmental window where the epigenome may be especially vulnerable to environmental and behavioural influence. Further, uncovering associations between sleep timing and dietary patterns with circadian genes could have important implications for later disease risk, particularly cardiometabolic health, as circadian genes play an important role in metabolism [16]. Within a pilot study of 142

Mexican adolescents, the primary aim was to assess whether weekday sleep midpoint, defined as the median of bed and wake time, was associated with DNA methylation at multiple CpG sites of 12 selected core circadian genes, quantified via the Infinium MethylationEPIC. These genes were selected for study due to their known function in regulating circadian rhythms. A secondary aim was to examine associations between dietary patterns and circadian gene methylation.

Results

Mean (SD) age of the sample was 14.0 (2.0) years, and 49% were male. Mean (SD) weekday midpoint was 3:41 AM (1.3 h). Later sleep midpoint was associated with older age and longer sleep duration (Table 1). Dietary pattern scores were not statistically significantly associated with midpoints, although adolescents with later midpoints tended to have lower intake of the *Plant-based and lean proteins* pattern and the *Eggs, milk & refined grain* pattern.

Overall, we examined associations with DNA methylation at 548 CpG sites within the 12 selected circadian genes. The statistically significant associations (at $P < 0.05$) between individual loci and the sleep midpoint and dietary pattern exposures are shown in Table 2.

Sleep midpoint and DNA methylation results

There were 24 associations with $P < 0.05$, representing loci across 8 different genes, and there were 3 associations at the Bonferroni-corrected P value of

Table 1. Characteristics of the 142 Mexican adolescents.

Sociodemographic and lifestyle characteristics	Midpoint <4 AM, mean (SD)	Midpoint ≥4 AM, mean (SD)	P value
N	87	55	
Age	13.6 (1.9)	14.5 (1.9)	0.006
Sex (%)			0.35
Male	65.2	34.8	
Female	57.5	42.5	
BMI for age z score	0.51 (1.20)	0.57 (1.36)	0.76
Mother's education (years)	11.0 (2.9)	11.1 (2.9)	0.80
Physical activity, moderate/vigorous minutes/day	84.1 (27.2)	82.4 (32.1)	0.73
Total energy intake (kcal)	2514 (1062)	2279 (873)	0.17
Dietary pattern scores			
Diet pattern 1: <i>Plant-based and lean proteins</i>	0.21 (1.28)	0.07 (1.33)	0.54
Diet pattern 2: <i>Meat & starchy foods</i>	0.06 (1.08)	0.03 (0.87)	0.88
Diet pattern 3: <i>Eggs, milk & refined grain</i>	0.14 (0.72)	-0.07 (0.98)	0.14
Weekday sleep duration (min)	497 (59)	542 (59)	<0.0001

Table 2. Associations between sleep and dietary patterns and DNA methylation of circadian genes in Mexican adolescents, $P < 0.05$.

Probe	Chromosome	Position	Gene	Relation to island	Gene region feature category	Beta values ¹	P value
<i>Sleep Midpoint (median of bed and wake time)</i>							
cg15603424	chr11	13300592	BMAL1	Island	5 UTR	0.000820	0.00300*
cg11591729	chr4	56411904	CLOCK	N_Shore	1stExon	0.000857	0.0318
cg14618246	chr11	45888058	CRY2	OpenSea	Body	0.00169	0.0253
cg25904177	chr11	45884565	CRY2	OpenSea	Body	-0.00178	0.0307
cg25736892	chr11	45888911	CRY2	OpenSea	Body	-0.00147	0.0450
cg13476336	chr17	38255694	NR1D1	Island	Body	0.00112	0.00564
cg06474323	chr17	38257020	NR1D1	Island	TSS200	0.000217	0.0185
cg02923856	chr3	23989124	NR1D2	S_Shore	5 UTR	0.00383	0.0302
cg10669351	chr3	24007477	NR1D2	OpenSea	Body	0.00301	0.0305
cg03301481	chr17	8052075	PER1	N_Shelf	Body	-0.00291	0.00360*
cg13433366	chr17	8057058	PER1	S_Shore	TSS1500	0.00292	0.0263
cg25250717	chr15	61144420	RORA	OpenSea	Body	-0.00207	0.0150
cg23965982	chr15	61055848	RORA	OpenSea	Body	0.00286	0.0154
cg18136062	chr15	61520931	RORA	Island	Body	0.000990	0.0156
cg09859112	chr15	61520370	RORA	N_Shore	Body	0.00170	0.0217
cg07701191	chr15	61520424	RORA	Island	Body	0.000502	0.0223
cg12061096	chr15	61028241	RORA	OpenSea	Body	0.00320	0.0260
cg02775377	chr15	61476554	RORA	OpenSea	Body	0.00232	0.0272
cg22536351	chr15	61044565	RORA	OpenSea	Body	0.00246	0.0388
cg20525725	chr15	61497556	RORA	OpenSea	Body	-0.00145	0.0488
cg13301933	chr15	61521439	RORA	Island	5 UTR	0.000819	0.0126
cg26087678	chr15	60884119	RORA	Island	Body	0.00328	0.0309
cg08700690	chr15	60884630	RORA	Island	Body	0.000523	0.0455
cg14170313	chr9	77112896	RORB	Island	1stExon	0.00109	0.00116*
<i>Diet pattern 1: Plant-based and lean proteins</i>							
cg06116839	chr11	13376988	BMAL1	OpenSea	Body	-0.00125	0.0340
cg19429538	chr4	56303887	CLOCK	OpenSea	Body	-0.00184	0.0400
cg02359589	chr17	8045663	PER1	OpenSea	Body	-0.00225	0.0495
cg07719617	chr2	239169696	PER2	OpenSea	Body	-0.00209	0.0102
cg21462970	chr2	239161488	PER2	OpenSea	Body	-0.00118	0.0148
cg06246651	chr2	239187798	PER2	OpenSea	5 UTR	-0.00325	0.0390
cg13469320	chr2	239162122	PER2	OpenSea	Body	-0.000987	0.0474
cg04917262	chr1	7868469	PER3	OpenSea	Body	-0.00414	0.000601*
cg11641053	chr1	7844184	PER3	Island	TSS1500	-0.000426	0.0293
cg13146107	chr15	61122353	RORA	OpenSea	Body	-0.00115	0.0286
cg05912053	chr15	60919520	RORA	OpenSea	Body	-0.00191	0.0265
cg20667664	chr17	38254448	NR1D1	N_Shore	Body	-0.00323	0.0207
<i>Diet pattern 2: Meat & starchy foods</i>							
cg16259330	chr11	13299070	BMAL1	Island	TSS1500	0.00109	0.00395*
cg21078679	chr11	13377829	BMAL1	OpenSea	Body	-0.00216	0.0237
cg03327818	chr11	13370408	BMAL1	OpenSea	5 UTR	0.00219	0.0271
cg27298069	chr4	56412099	CLOCK	Island	TSS200	-0.000441	0.0145
cg16345521	chr12	107486801	CRY1	Island	1stExon	0.000713	0.00535
cg26125082	chr11	45868418	CRY2	N_Shore	TSS1500	0.00942	0.0229
cg20855344	chr3	2398090	NR1D2	N_Shore	TSS1500	-0.00301	0.0332
cg08506046	chr3	23987101	NR1D2	Island	TSS1500	0.00105	0.0369
cg21093368	chr17	8055834	PER1	Island	TSS200	-0.00144	0.0459
cg03067929	chr15	61191921	RORA	OpenSea	Body	0.00607	0.0253
cg26065596	chr15	61448727	RORA	OpenSea	Body	-0.00198	0.0351
cg03461216	chr15	61197849	RORA	OpenSea	Body	-0.00278	0.0380
cg04324336	chr15	60941269	RORA	OpenSea	Body	0.00218	0.0431
cg19170589	chr15	60883569	RORA	Island	Body	0.00189	0.000658*
cg14331163	chr9	77111663	RORB	N_Shore	TSS1500	0.00321	0.0471
<i>Diet pattern 3: Eggs, milk & refined grain</i>							
cg22322535	chr11	13299888	BMAL1	Island	5 UTR	-0.00118	0.0184
cg13250711	chr11	13298675	BMAL1	N_Shore	TSS1500	-0.00139	0.0211
cg07032258	chr11	13357037	BMAL1	OpenSea	5 UTR	-0.00448	0.0328
cg08367205	chr11	13298296	BMAL1	N_Shore	TSS1500	-0.00102	0.0356
cg18579796	chr11	13371358	BMAL1	OpenSea	5 UTR	0.00285	0.00229*
cg14666553	chr11	13374140	BMAL1	OpenSea	5 UTR	0.00198	0.0125
cg16839955	chr11	13298683	BMAL1	N_Shore	TSS1500	-0.00119	0.0164
cg21860398	chr11	13379485	BMAL1	OpenSea	Body	-0.00271	0.0212
cg16340122	chr11	13377690	BMAL1	OpenSea	Body	0.00389	0.0447
cg02683371	chr4	56298893	CLOCK	OpenSea	3 UTR	0.00204	0.0441
cg24000108	chr4	56376331	CLOCK	OpenSea	5 UTR	0.00373	0.0174
cg12893431	chr4	56315641	CLOCK	OpenSea	Body	0.00450	0.0196
cg24004414	chr4	56369919	CLOCK	OpenSea	5 UTR	0.00674	0.0232

(Continued)

Table 2. (Continued).

Probe	Chromosome	Position	Gene	Relation to island	Gene region feature category	Beta values ¹	P value
cg15048607	chr4	56310021	<i>CLOCK</i>	OpenSea	Body	0.00526	0.0262
cg19429538	chr4	56303887	<i>CLOCK</i>	OpenSea	Body	0.00288	0.0394
cg08407670	chr4	56303880	<i>CLOCK</i>	OpenSea	Body	0.00273	0.0442
cg26520722	chr12	107486229	<i>CRY1</i>	N_Shore	Body	-0.00240	0.00536
cg08756366	chr12	107444216	<i>CRY1</i>	OpenSea	Body	0.00308	0.00749
cg20879606	chr11	45868932	<i>CRY2</i>	Island	Body	-0.00181	0.00831
cg07524456	chr11	45868501	<i>CRY2</i>	N_Shore	TSS1500	-0.00941	0.0321
cg14858951	chr17	38257028	<i>NR1D1</i>	Island	TSS200	-0.000435	0.0134
cg01059731	chr17	38255663	<i>NR1D1</i>	Island	Body	-0.00230	0.0152
cg13476336	chr17	38255694	<i>NR1D1</i>	Island	Body	-0.00130	0.0205
cg20667664	chr17	38254448	<i>NR1D1</i>	N_Shore	Body	0.00471	0.0303
cg04534726	chr3	23987467	<i>NR1D2</i>	Island	TSS200	-0.000452	0.00999
cg17680792	chr3	23986459	<i>NR1D2</i>	N_Shore	TSS1500	-0.000750	0.0282
cg19399789	chr3	23987441	<i>NR1D2</i>	Island	TSS200	-0.000492	0.00869
cg06103301	chr3	23987730	<i>NR1D2</i>	Island	5 UTR	-0.000306	0.00991
cg10028884	chr17	8054829	<i>PER1</i>	Island	5 UTR	-0.00111	0.0166
cg03301481	chr17	8052075	<i>PER1</i>	N_Shelf	Body	0.00303	0.0299
cg20503576	chr17	8055884	<i>PER1</i>	S_Shore	TSS200	-0.00199	0.0440
cg13469320	chr2	239162122	<i>PER2</i>	OpenSea	Body	0.00233	0.00234*
cg12308675	chr2	239169537	<i>PER2</i>	OpenSea	Body	-0.00298	0.00290
cg02976543	chr2	239197607	<i>PER2</i>	Island	TSS1500	-0.00616	0.0138
cg06174546	chr2	239171720	<i>PER2</i>	OpenSea	Body	0.00152	0.0247
cg00811891	chr1	7904830	<i>PER3</i>	OpenSea	3 UTR	0.00204	0.0409
cg11129701	chr1	7862851	<i>PER3</i>	OpenSea	5 UTR	-0.00203	0.0449
cg22148904	chr1	7862234	<i>PER3</i>	OpenSea	5 UTR	0.00392	0.0498
cg27391826	chr15	61395262	<i>RORA</i>	OpenSea	Body	0.00616	0.00516
cg08574592	chr15	61520873	<i>RORA</i>	Island	Body	-0.00199	0.0112
cg18760360	chr15	60953215	<i>RORA</i>	OpenSea	Body	0.00409	0.0121
cg02076826	chr15	61477299	<i>RORA</i>	OpenSea	Body	-0.00395	0.0174
cg20822028	chr15	61038077	<i>RORA</i>	OpenSea	Body	-0.00256	0.0182
cg27024408	chr15	61327619	<i>RORA</i>	OpenSea	Body	0.00285	0.0184
cg18730873	chr15	61462794	<i>RORA</i>	OpenSea	Body	0.00276	0.0232
cg27077827	chr15	60943536	<i>RORA</i>	OpenSea	Body	-0.00272	0.0311
cg13146553	chr15	61343563	<i>RORA</i>	OpenSea	Body	-0.00444	0.0320
cg12061096	chr15	61028241	<i>RORA</i>	OpenSea	Body	-0.00416	0.0367
cg05673940	chr15	61334658	<i>RORA</i>	OpenSea	Body	-0.00241	0.0390
cg12643950	chr15	61210235	<i>RORA</i>	OpenSea	Body	-0.00273	0.0401
cg00237177	chr15	61233587	<i>RORA</i>	OpenSea	Body	0.00134	0.0444
cg21756476	chr15	61182765	<i>RORA</i>	OpenSea	Body	0.00716	0.0491
cg11227596	chr15	61029537	<i>RORA</i>	OpenSea	Body	-0.00269	0.0495
cg01691639	chr15	61521458	<i>RORA</i>	Island	5 UTR	-0.00204	0.00293*
cg05334993	chr15	60888221	<i>RORA</i>	S_Shelf	Body	0.00246	0.0158
cg19170589	chr15	60883569	<i>RORA</i>	Island	Body	-0.00165	0.0142
cg18064474	chr15	60885021	<i>RORA</i>	Island	Body	-0.000368	0.0499
cg04600961	chr15	60787182	<i>RORA</i>	OpenSea	3 UTR	0.00681	0.0381
cg14736496	chr15	60920638	<i>RORA</i>	OpenSea	TSS1500	0.00668	0.00822
cg09094797	chr9	77230369	<i>RORB</i>	OpenSea	Body	-0.00658	0.0469
cg09363447	chr1	151787446	<i>RORC</i>	OpenSea	Body	0.00528	0.00180*
cg25206113	chr1	151788262	<i>RORC</i>	OpenSea	Body	0.00679	0.0404

¹Adjusted for sex, age, and % monocyte.

*Meets Bonferroni-corrected P value threshold (P < 0.004).

<0.004. These included a positive association between midpoint and cg14170313, a CpG site within the island region of *RORB* (P = 0.001), a positive association with cg15603424, a CpG site within an island of *BMAL1* at the 5' UTR (P = 0.003), and an inverse association with cg03301481, a site found within the gene body of *PER1* (P = 0.0036).

Due to the fact that some circadian genes had a higher coverage (*RORA*, in particular, had 192 CpG sites while the remaining genes had between

19 and 64), we next calculated the proportion of loci within a particular gene that had statistically significant associations (P < 0.05). Several genes were found to have >5% of CpG sites with statistically significant associations (Table 3). To illustrate, later midpoint was associated with differential methylation of 11% of the CpG sites measured within *PER1* and 9% with both *CRY2* and *NR1D1*; and the majority of these associations were in the positive direction.

Next, we averaged DNA methylation levels across CpG sites within annotated CpG islands (some genes had coverage at multiple CpG islands). For sleep midpoint, there were positive associations between DNA methylation of *RORA* island 3 ($\beta = 0.018$; $P = 0.045$) and for *RORB* ($\beta = 0.04$; $P = 0.003$). In general, most associations between sleep midpoint and island regions were positively correlated (Table 4).

To ascertain whether any of the sleep midpoint findings could be attributed to sleep duration (since sleep midpoint and sleep duration were positively correlated), we ran sensitivity analyses using sleep duration as the outcome. We found essentially no overlap in the associations, indicating that sleep midpoint results were independent of sleep duration.

Diet pattern results

For *Plant-based and lean proteins* pattern, there were 12 associations at $P < 0.05$ across 7 genes with 1 at the Bonferroni-corrected threshold; the *Meat & starchy foods* diet pattern had 15 associations at $P < 0.05$ across 7 genes and 2 at the Bonferroni-corrected threshold; and the *Eggs, milk & refined grain* pattern had 62 associations at $P < 0.05$ from all 12 genes, and 4 at the Bonferroni-corrected threshold. Overall, the two most statistically significant associations (smallest P values) were for cg04917262 (found in the gene body of *PER3*) and the *Plant-based and lean proteins* diet pattern ($P = 0.0006$); and for cg19170589 (found in an island region of *RORA*) with the *Meat & starchy foods* diet pattern ($P = 0.0007$). Focusing in on the loci found in islands, higher adherence to the *Plant-based and lean proteins* diet pattern was associated with differential methylation in 10% of the *PER2* CpG island loci, associations that were generally in the inverse direction. Multiple genes were highlighted in the *Eggs, milk & refined grain* pattern: *BMAL1*, *NR1D1*, *NR1D2*, *PER1*, and *PER2* each had higher than 10% of CpG sites that were associated at $P < 0.05$, and in an inverse direction. Of these, 19% of the loci within *NR1D1* were associated with the *Eggs, milk & refined grain* pattern.

Next, examining the associations between dietary patterns and the averaged CpG sites within

island regions (Table 4), we found one positive association between the *Plant-based and lean proteins* diet pattern and *PER1*. There were no associations for *Meat & starchy foods* pattern. For the *Eggs, milk & refined grain* pattern, there were inverse associations for *PER2*, *CRY2*, *RORA* island 1, *RORA* island 3, and *NR1D1* island 1. In general, most associations between the *Eggs, milk & refined grain* and island regions were inversely correlated, while directions of associations with the other dietary patterns were mixed.

Post-hoc analyses

Supplemental exploratory sex-stratified analyses revealed that many of the associations between sleep/dietary exposures and circadian gene CpG islands varied by sex (Table 4). For example, associations with sleep midpoint were more evident among girls. For dietary patterns, some associations (e.g., island of *PER1* with the *Plant-based and lean proteins* pattern) were more evident among boys while others were more apparent among girls (e.g., island of *PER2* with the *Eggs, milk & refined grain* pattern). Nonetheless, these analyses were underpowered.

Finally, we conducted post-hoc analysis among a subsample of participants who had RNA-seq data (Supplemental Table 1). We found weak inverse correlations, although not statistically significant, between island DNA methylation and gene expression in *PER2* (-0.13), *PER3* island 1 (-0.11), and *RORB* (-0.12). We found weak positive correlations between island DNA methylation and gene expression in *BMAL1* (0.19), *RORA* island 3 (0.36), *NR1D1* island 2 (0.14), and *NR1D2* (0.27). The only correlation that was statistically significant was the positive correlation with *RORA* island 3.

Discussion

Within this cohort of Mexican adolescents, delayed sleep timing was generally associated with higher DNA methylation of core clock genes, whereas higher intake of a dietary pattern composed of breakfast foods was associated with lower methylation of core clock genes. In particular, actigraphy-assessed sleep timing was

Table 3. Associations between sleep and dietary patterns and DNA methylation of circadian genes in Mexican adolescents, overall description.

	Number of probes in gene	Proportion of probes with association $P < 0.05$	Number of probes in islands	Proportion of island probes with $P < 0.05$	Direction of island probe association (majority)
Sleep Midpoint (median of bed and wake time)					
<i>BMAL1</i>	64	0.016	9	0.11	+
<i>CLOCK</i>	43	0.023	13	0	
<i>CRY1</i>	28	0	11	0	
<i>CRY2</i>	32	0.094	8	0	
<i>NR1D1</i>	22	0.091	12	0.17	+
<i>NR1D2</i>	26	0.077	10	0	
<i>PER1</i>	19	0.11	5	0	
<i>PER2</i>	39	0	6	0	
<i>PER3</i>	34	0	15	0	
<i>RORA</i>	192	0.063	28	0.18	+
<i>RORB</i>	26	0.038	4	0.25	+
<i>RORC</i>	23	0	0	0	
Diet pattern 1: Plant-based and lean proteins					
<i>BMAL1</i>	64	0.016	9	0	
<i>CRY1</i>	28	0	11	0	
<i>CRY2</i>	32	0	8	0	
<i>CLOCK</i>	43	0.023	13	0	
<i>NR1D1</i>	21	0.048	11	0	
<i>NR1D2</i>	26	0	10	0	
<i>PER1</i>	19	0.053	5	0.2	-
<i>PER2</i>	39	0.103	6	0	
<i>PER3</i>	34	0.059	15	0.07	-
<i>RORA</i>	192	0.010	28	0.04	-
<i>RORB</i>	26	0	4	0	
<i>RORC</i>	23	0	0	0	
Diet pattern 2: Meat & starchy foods					
<i>BMAL1</i>	64	0.047	9	0.11	+
<i>CLOCK</i>	43	0.023	13	0.08	-
<i>CRY1</i>	28	0.036	11	0.09	+
<i>CRY2</i>	32	0.031	8	0	
<i>NR1D1</i>	21	0	11	0	
<i>NR1D2</i>	26	0.077	10	0.1	+
<i>PER1</i>	19	0.053	5	0.2	-
<i>PER2</i>	39	0	6	0	
<i>PER3</i>	34	0	15	0	
<i>RORA</i>	192	0.026	28	0.04	+
<i>RORB</i>	19	0.053	4	0	
<i>RORC</i>	23	0	0	0	
Diet pattern 3: Eggs, milk & refined grain					
<i>CLOCK</i>	43	0.163	13	0	
<i>BMAL1</i>	64	0.141	9	0.11	-
<i>CRY1</i>	28	0.071	11	0	
<i>CRY2</i>	32	0.063	8	0.13	-
<i>NR1D1</i>	21	0.190	11	0.27	-
<i>NR1D2</i>	26	0.154	10	0.3	-
<i>PER1</i>	19	0.158	5	0.2	-
<i>PER2</i>	39	0.103	7	0.17	-
<i>PER3</i>	34	0.088	15	0	
<i>RORA</i>	192	0.005	28	0.14	-
<i>RORB</i>	26	0.038	4	0	
<i>RORC</i>	23	0.087	0	0	

associated with DNA methylation of several core circadian genes, notably with *RORB*, *PER1*, *CRY2*, and *NR1D1*. Each of the three dietary patterns examined was also related to DNA methylation

patterns, but the *Eggs, milk & refined grain* pattern (i.e., breakfast pattern) had the clearest evidence of relationships with circadian genes, with associations across all 12 genes. Within the CpG island

Table 4. Associations between sleep and dietary patterns and DNA methylation of circadian gene CpG islands in adolescents.

	All		Boys		Girls	
	Beta coefficient ¹	P value	Beta coefficient ²	P value	Beta coefficient ²	P value
Sleep Midpoint (median of bed and wake time)						
<i>PER1</i>	.0140337	0.240	-.0102469	0.550	.0345147	0.042
<i>PER2</i>	.0040428	0.615	-.0017354	0.893	.0081995	0.421
<i>PER3</i> island 1	-.0081581	0.664	-.0257108	0.286	.0083659	0.773
<i>PER3</i> island 2	-.001583	0.848	.0043872	0.734	-.0082874	0.445
<i>PER3</i> island 3	.0774699	0.096	.0523266	0.464	.1028792	0.099
<i>CRY2</i>	.0029527	0.711	.0088452	0.400	-.0039388	0.742
<i>BMAL1</i>	.0117632	0.140	.0107429	0.327	.0114565	0.331
<i>CLOCK</i>	.0069998	0.298	.0026318	0.766	.0101371	0.320
<i>RORA</i> island 1	.0095559	0.204	.0031786	0.754	.0131733	0.233
<i>RORA</i> island 2	.0074266	0.442	-.0092649	0.503	.0215126	0.119
<i>RORA</i> island 3	.0175065	0.045	.0039397	0.768	.0281399	0.014
<i>RORB</i>	.0353199	0.003	.0372594	0.050	.0330129	0.030
<i>NR1D1</i> island 1	.0127016	0.244	.0116515	0.471	.0151853	0.317
<i>NR1D1</i> island 2	.0080007	0.360	.0180043	0.134	-.0024247	0.846
<i>NR1D2</i>	.0110481	0.104	.0072648	0.448	.0134684	0.172
Diet pattern 1: Plant-based and lean proteins						
<i>PER1</i>	.0008977	0.023	.00123	0.007	.0002354	0.751
<i>PER2</i>	.0000904	0.791	-.0000792	0.857	.0005708	0.305
<i>PER3</i> island 1	.0004076	0.408	.0003283	0.517	.0006691	0.498
<i>PER3</i> island 2	.0000113	0.978	-.0001049	0.830	.0001876	0.807
<i>PER3</i> island 3	-.0002882	0.939	-.002409	0.649	.0035876	0.520
<i>CRY2</i>	.0000126	0.935	.0001874	0.296	-.0003008	0.281
<i>BMAL1</i>	-.0001708	0.330	.0001164	0.562	-.0007621	0.019
<i>CLOCK</i>	.0000122	0.893	.000103	0.314	-.0001479	0.386
<i>RORA</i> island 1	-.0000955	0.693	.0000768	0.783	-.0003675	0.400
<i>RORA</i> island 2	-.0001234	0.339	.0000478	0.760	-.0004734	0.039
<i>RORA</i> island 3	.0000297	0.864	.0002662	0.216	-.0003976	0.176
<i>RORB</i>	-.0003219	0.282	-.000339	0.387	-.0003028	0.544
<i>NR1D1</i> island 1	.0002256	0.509	.0006367	0.127	-.0006509	0.281
<i>NR1D1</i> island 2	-.00004	0.766	.0000442	0.779	-.0001438	0.542
<i>NR1D2</i>	.0000639	0.514	.0000652	0.547	.0000771	0.681
Diet pattern 2: Meat & starchy foods						
<i>PER1</i>	.0001683	0.745	.0007263	0.373	-.0001832	0.792
<i>PER2</i>	-.0004792	0.277	-.0003113	0.686	-.0007297	0.161
<i>PER3</i> island 1	.000674	0.291	.0011211	0.203	.0003541	0.703
<i>PER3</i> island 2	-.0005741	0.285	-.0008578	0.314	-.0003475	0.629
<i>PER3</i> island 3	-.0002193	0.964	-.0025248	0.785	.0015882	0.762
<i>CRY2</i>	-.0000955	0.632	.0001579	0.616	-.0003013	0.250
<i>BMAL1</i>	.000112	0.623	.0003808	0.277	-.0000548	0.860
<i>CLOCK</i>	.0000891	0.450	.0003705	0.036	-.0001049	0.513
<i>RORA</i> island 1	.0002087	0.506	.0003642	0.455	4.67e-06	0.991
<i>RORA</i> island 2	.0000125	0.940	.0003688	0.175	-.0002097	0.336
<i>RORA</i> island 3	-.0000466	0.836	.0002262	0.550	-.0002784	0.314
<i>RORB</i>	.0003113	0.422	-.0002669	0.698	.0006562	0.158
<i>NR1D1</i> island 1	-.0001372	0.757	.0001722	0.815	-.0002654	0.641
<i>NR1D1</i> island 2	-.0000136	0.938	.0002066	0.452	-.0002197	0.320
<i>NR1D2</i>	.0000413	0.745	.0001992	0.291	-.0000729	0.679
Diet pattern 3: Eggs, milk & refined grain						
<i>PER1</i>	-.0010701	0.083	-.0013847	0.082	-.0006233	0.533
<i>PER2</i>	-.0012047	0.022	-.0004974	0.511	-.0019366	0.008
<i>PER3</i> island 1	-.0002299	0.764	.0001473	0.866	-.0007279	0.585
<i>PER3</i> island 2	-.0006967	0.280	-.001359	0.102	3.43e-07	1.000
<i>PER3</i> island 3	-.0031526	0.588	-.0033161	0.716	-.0035995	0.632
<i>CRY2</i>	-.0005672	0.017	-.0006632	0.029	-.0003686	0.328
<i>BMAL1</i>	-.0005028	0.064	-.0006093	0.075	-.000339	0.447
<i>CLOCK</i>	-.0001487	0.293	-.0001681	0.340	-.000082	0.722
<i>RORA</i> island 1	-.0009331	0.012	-.0006093	0.202	-.0011282	0.053
<i>RORA</i> island 2	-.0002716	0.175	-.0006511	0.013	.0002449	0.435
<i>RORA</i> island 3	-.0006814	0.010	-.000516	0.163	-.0007874	0.045
<i>RORB</i>	-.0004259	0.360	-.0003936	0.560	-.000444	0.509
<i>NR1D1</i> island 1	-.0010925	0.038	-.0017573	0.013	-.0004323	0.597
<i>NR1D1</i> island 2	-.0003233	0.120	-.000532	0.046	.0000399	0.900
<i>NR1D2</i>	-.0002782	0.066	-.0002651	0.152	-.0002584	0.305

¹Adjusted for sex, age, and % monocytes.²Adjusted for age and % monocytes.

regions, there were inverse associations between the *Eggs, milk & refined grain* pattern and *PER2*, *CRY2*, *RORA*, and *NR1D1*.

Prior studies examining the role of sleep on circadian gene DNA methylation patterns are rare, although a few adult studies exist. In line with the direction of the present findings, DNA methylation of core clock genes (candidate gene analysis) showed that sleep deprivation among nurses and midwives was associated with higher DNA methylation of *PER2* [17], although it was associated with lower methylation of *CRY2*. The study did not examine the role of sleep timing, although sleep deprivation is likely correlated with misaligned sleep times, especially in this population of workers who often work night shifts. Another study of 124 health-care workers found higher methylation of *PER3* (at three loci within the gene body) in nightshift workers compared to dayshift workers [14]. An experimental investigation of sleep deprivation and circadian gene methylation and expression among 15 young men found that in response to sleep deprivation, there was higher methylation of *CRY1* and *PER1* in adipose tissue, but no differentially methylated sites in skeletal tissue [18]. Differences in DNA methylation across tissues may help explain inconsistencies between studies that use leukocyte DNA and DNA from other tissues.

Associations between the *Eggs, milk & refined grain* pattern and circadian gene DNA methylation were particularly notable as they were more numerous than associations with sleep or the other dietary patterns. Furthermore, these associations were consistently inverse, which was opposite the direction observed for sleep timing. There may be a few explanations as to why the associations with this dietary pattern were robust and consistent. The *Eggs, milk & refined grain* pattern contains breakfast foods, and thus could be a proxy for the regular consumption of breakfast. The daily timing of eating, which begins with breakfast, is an important circadian regulator and may also have effects on metabolic health [19,20]. The nutrients abundant within foods comprising the *Eggs, milk & refined grain* dietary pattern may also play a role. Eggs and fortified grains are sources of folate, a nutrient that can play a role in maintaining DNA methylation profiles following mitosis [21,22]. In addition, eggs and

dairy contain nutrients that feed the one-carbon metabolism pathway which is also important for maintaining DNA methylation, including vitamin B12, choline, and methionine [23]. Associations with the other two dietary patterns, the *Plant-based and lean proteins* and the *Meat & starchy* pattern, were much fewer in number. Yet, the direction of the associations with the *Plant-based and lean proteins* pattern, also rich in methyl donors such as folate and vitamin B12, was in the same direction as the *Eggs, milk & refined grain* pattern (inverse), while the associations with the *Meat & starchy* pattern were more mixed.

Overall, there was evidence that delayed sleep timing and diet patterns (in particular the breakfast pattern) were associated with DNA methylation of circadian genes in adolescents. The fact that these associations could be detected in a healthy population of adolescents is enlightening, because it indicates that even relatively constrained variability in sleep times and dietary patterns (as opposed to complete circadian shifts found in shift work populations) are associated with differences in circadian gene DNA methylation patterns. Due to the cross-sectional study design, it cannot be determined whether lifestyle behaviours preceded DNA methylation or vice versa. However, these findings provide preliminary support for a potential pathway whereby delayed sleep timing and regular consumption of breakfast foods are related to DNA methylation of circadian genes, which could ultimately affect gene expression and phenotypic outcomes such as BMI. Increasing evidence exists for associations between circadian gene methylation and BMI [24,25], although no studies have yet linked sleep, diet, DNA methylation, and BMI in a longitudinal manner. Another unique finding is that the associations may differ by sex. Of note was that associations with sleep midpoint were more pronounced (larger effect sizes and/or higher precision) among girls. Although speculative, it is worth mentioning that later midpoints among girls could be reflective of chronic insomnia, a sleep disorder that is much more prevalent among females than males [26], and which might have independent associations with DNA methylation [27].

In exploratory post-hoc analysis, we examined whether DNA methylation of these circadian genes was associated with gene expression in

a subset of the participants. While this analysis was statistically underpowered, we observed correlations in the expected inverse direction for *PER2*, *PER3* island 1, and *RORB*. In contrast, we also observed some positive correlations, with the strongest for *RORA* island 3 (the only statistically significant correlation) and *NR1D2*. These results provide some preliminary evidence that DNA methylation of these circadian genes could affect gene expression, although the magnitudes of these correlations are relatively low. The inconsistent directions of the correlations could also have to do with the fact that not all of the CpG islands were located within promoter regions (e.g., *RORA* island 3 was found in the gene body).

Strengths of the study include the use of objective data (from actigraphy) to characterize week-day sleep timing, and the ability to examine multiple important circadian genes. This pilot analysis had a limited sample size; thus, findings warrant replication in larger study populations and in populations from different ethnic backgrounds. Given that the study sample was reflective of a low to middle-SES population in Mexico City, the findings may also not be broadly generalizable to the entire Mexican population. Further, there may be unmeasured confounders, such as mental health and stress-related factors, which can be associated both with DNA methylation and with disrupted sleep and eating patterns [28]. The ability to complete fully powered sex-stratified analyses would also elucidate whether findings indeed differed for boys versus girls (as they appeared in exploratory analyses). The fact that the samples were leukocytes from whole blood also means we could not investigate DNA methylation within other tissues of interest for cardiometabolic health and/or sleep regulation such as brain, liver, or adipose tissue which were not feasible to collect in our study population. Finally, there was not access to chronotype information (whether adolescents were more ‘morning’ types or ‘evening’ types).

In summary, within a sample of Mexican adolescents, there were cross-sectional associations between sleep timing and eating patterns with DNA methylation of core circadian genes. Future work that links sleep, diet, and DNA methylation

with longitudinal change in metabolic outcomes will be pivotal to understanding any potential effects of misaligned sleep and eating on long-term metabolic health in adolescents.

Methods

Study population

The study sample included adolescent participants from 2 of 3 sequentially enrolled cohorts of the Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT) study [29]. Between 1997 and 2004, 1012 mother/child dyads were recruited from prenatal clinics of the Mexican Social Security Institute in Mexico City, which serves low- to middle-income populations formally employed in the private sector. At baseline, mothers reported sociodemographic and health information. Children were followed from birth approximately every 6–12 months to 5 years of age and then periodically over mid-childhood and adolescence to gain information on growth and relevant environmental and nutritional exposures. In 2015, a subset of 550 participants from the original birth cohorts 2 and 3, who were in the midst of pubertal transition (ages 9 to 17 years), were selected to participate in a follow-up visit. The present analysis includes adolescents who took part in this 2015 follow-up visit ($n = 550$), held in a research facility of the ABC hospital of Mexico City. Within a pilot sample of 145 adolescents (selected for analysis based on availability of biospecimens and completeness of prior visits), epigenome-wide analyses were conducted with blood leukocyte DNA taken during the follow-up visit. Of these, 142 adolescents also had 7-day actigraphy collected for sleep estimation, taken in the week following the in-clinic visit. The institutional review boards at the Mexico National Institute of Public Health and the University of Michigan approved the research protocols. Informed consent was obtained from parents for all participants in addition to participant assent.

Sleep timing

At the end of the clinical follow-up, adolescents were given an actigraph (ActiGraph GT3X+;

ActiGraph LLC, Pensacola, FL) to wear on their non-dominant wrist continuously for 7 days. Nightly sleep parameters were estimated from the actigraphic data with the use of a fusedLASSO (least absolute shrinkage and selection operator)-based calculator package developed in R (R Foundation for Statistical Computing, Vienna) [30]. The primary exposure of the study was average weekday midpoint. Midpoint refers to the median of sleep onset and wake time (reported in decimal hours), and a later sleep midpoint is considered a proxy for circadian misalignment.

Diet patterns

During the adolescent follow-up visit, a trained interviewer administered a 116-item semi-quantitative food frequency questionnaire to the adolescents to obtain information on typical consumption habits. The questionnaire asked adolescents to recall how often they had consumed one serving of a standard portion size of each food item over the previous week [31]. Photographs of food items were provided as a visual aid. Response options ranged from never to ≥ 6 times per day. We converted the raw response values (1–9) into servings/day. Dietary patterns were identified in a previous analysis using principal component analysis [32]. Each adolescent receives a score representing adherence to the dietary patterns, with higher scores representing higher similarity to the pattern. Three dietary patterns were identified in this cohort; the first pattern, called the *Plant-based & lean proteins* pattern, was characterized by high intake frequency of vegetables, fruit, soup, fish, water and unsweetened drinks, and high-fat dairy. The second pattern, called *Meat & starch*, was marked by high intake frequency of ‘Western’ processed foods including chips, refined grains, sugar-sweetened beverages, processed meat, and high-fat dairy, as well as consumption more in line with ‘traditional’ foods such as the Mexican food group (e.g., tacos and quesadillas), potatoes and fried plantains, soup, legumes, and corn tortillas. The third pattern, the *Eggs, milk & refined grain* pattern, was characterized by high intake frequency of refined grains, milk, sweetened milk, mayonnaise or margarine, and eggs.

DNA methylation

Whole blood was collected via venipuncture and stored in EDTA-preserved at -80°C prior to processing. DNA was isolated from blood leukocytes via the Flexigene kit (Qiagen). For epigenome-wide analysis of DNA methylation, blood leukocyte DNA samples were first treated with sodium bisulphite, which converts unmethylated cytosines to uracils leaving methylated cytosines unchanged [33]. DNA methylation profiles were then assessed with the Infinium MethylationEPIC BeadChip (Illumina). The BeadChip interrogates methylation at $>850,000$ cytosine-guanine dinucleotides (CpG sites) in gene promoters, enhancers, within gene bodies, and in intergenic regions [34]. Bisulphite converted samples were randomized across chips and chip positions, hybridized to BeadChips, and signals read and processed by experienced personnel in the University of Michigan Advanced Genomics Core. Data produced by the BeadChip were processed according to a pipeline designed for this technology and previously utilized [35]. Briefly, raw data consisting of average betas, the proportion of methylated cytosines at each site, were read into R Project for Statistical Computing using minfi [36]. Background correction, dye bias correction, and functional normalization to remove unwanted technical variation estimated from control probes included on each chip were performed [37,38]. Poorly detected probes (in at least 5% of samples), probes known to be cross-reactive [39], and probes with polymorphisms in the CpG site or the single base extension site were excluded. Cell type proportions from the blood leukocyte samples were estimated based on data from tissue-specific differentially methylated regions included on the BeadChip [40,41]. Final data consisted of beta values which are representative of the proportion methylated (from 0 to 1) at each CpG site. Data were extracted from all probes annotated of 12 circadian genes, including *BMAL1*, *CLOCK*, *CRY1*, *CRY2*, *NR1D1*, *NR1D2*, *PER1*, *PER2*, *PER3*, *RORA*, *RORB*, and *RORC*, with 548 total CpG sites (average of 46 CpG sites/gene, with a range of 19 to 192). These genes play an important role in

establishing circadian rhythms [42]. BMAL1 and CLOCK genes form the positive limb of a feedback loop and are expressed at night, while PER and CRY genes form the negative feedback loop and are expressed during the day. ROR genes and NR1D1/NR1D2 are nuclear receptors that are part of the stabilizing loop for the core feedback loop. Mutations in these genes can result in altered circadian phases and abnormal sleep patterning [43].

Gene expression analysis via RNA-sequencing

For a subset of participants with adequate RNA quality and quantity ($n = 72$), next-generation sequencing of RNA ('RNA-Seq') was completed to obtain relative expression data for all genes, and results were utilized from the 12 circadian genes of interest to this study. Following collection of whole blood into EDTA-containing tubes, white blood cells were extracted, preserved in RNALater, and stored frozen (-80°C) until further processing. RNA was isolated via the All-Prep kit (Qiagen). Quality and quantity were assessed via a Bioanalyzer Tapestation (Agilent). Libraries were prepared with Universal Plus mRNA-Seq with Human globin AnyDeplete (NuGEN Technologies, Inc.) which removes globin transcripts that are highly abundant in blood samples. Library preparation and sequencing were performed at the University of Michigan Advanced Genomics Core. Paired-end 50 cycle sequencing on an Illumina HiSeq 4000 was performed. Quality of the raw reads data for each sample was checked using FastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>; version 0.11.3). The Tuxedo Suite software package was used for alignment [44–46]. Briefly, reads were aligned to the reference mRNA transcriptome (hg19) (<http://genome.ucsc.edu/>) using TopHat (version 2.0.13) and Bowtie2 (version 2.2.1.) followed by a second round of post-alignment quality control in FastQC. One sample was dropped due to low alignment rates. All samples used in downstream analysis ($n = 71$) had at least 16.8 million good quality aligned reads. Prior to analysis, read counts were normalized by the trimmed mean of M-values method [47].

Covariates

Other covariates included in the present analysis were sex, age, BMI-for-age Z scores, maternal education, physical activity and percentage of monocytes (as a proportion of total leukocytes, described above). Trained research assistants measured height in cm (Tonelli E120 A) and weight in kg (InBody230). BMI Z scores (BMIZ) accounting for age and sex were calculated based on the World Health Organization reference [48]. Maternal education was reported by mothers at the original cohort enrolment visit and was categorized as <9 years, 9 to 11 years, 12 years, or >12 years of schooling. Physical activity was estimated from the accelerometers as average minutes of moderate/vigorous activity per day as previously described [49].

Statistical analysis

Means $\pm$ SD of sociodemographic and lifestyle characteristics were first estimated according to earlier versus later sleep midpoint (split at the median). Next, linear regression analyses were used to evaluate the associations between midpoint and each circadian gene CpG site, adjusting for age, sex, and % monocyte. Adjustment for other covariates did not affect results and thus were not retained. Sensitivity analyses with sleep duration as the outcome were also conducted, to determine whether results were similar for sleep midpoint and duration. Similar regression models were run for each dietary pattern score (all dietary patterns in separate models). Next, DNA methylation at annotated CpG island regions within these genes was examined. To do so, data from all of the CpG sites within a specific island region (some genes had more than one island region) were averaged (see Supplemental Table 1 for location of island sites and description of the location, i.e., promoter region versus gene body). Linear regression analysis was then conducted in the same manner as for the individual CpG sites, but with the averaged island regions as the outcomes. For these models, exploratory sex-stratified analyses were also run.

Finally, whether the % DNA methylation in the island regions corresponded to gene expression

(RNA-seq) was examined. Using a subset of participants ($N = 71$) that also had RNA-seq data, the correlations between the % methylation of the islands with RNA-seq data of the corresponding genes were obtained.

Due to the exploratory nature of the analyses, any findings at $P < 0.05$ are reported. A Bonferroni-corrected P -value threshold of $P < 0.004$ (0.05 divided by 12 genes) was also applied. All analyses were performed in Stata 14.0.

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Disclosure statement

Dr Chervin has served on member boards or advisory board for the American Academy of Sleep Medicine, Associated Professional Sleep Societies, International Pediatric Sleep Association, Sweet Dreamzzz, and the Pajama Program; and received royalties from UpToDate and Cambridge University Press. Authors report no conflicts of interest.

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